The Inhibition of Tumor Growth and Microvascular Angiogenesis by the Potent Angiogenesis inhibitor, TNP-470 in Rats(Dissertation_全文)

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庭野元孝

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主論文

The Inhibition of Tumor Growth and Microvascular Angiogenesis by the Potent Angiogenesis Inhibitor, TNP-470 in Rats

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Key Words: TNP-470, interstitial fluid pressure, vascular cast, sprout

Abstract : The antiangiogenic effects of TNP-470 on the neovascularization of tumors were studied by examining ultrastructural alterations in the vasculature and interstitial fluid pressure (IFP) of tumors. Wistar rats were first inoculated subcutaneously (sc) with the Walker 256 carcinosarcoma cell line, then either vehicle medium or TNP-470, 30mg/kg, was injected sc on day 1. A tumor growth assay, the necrotic area, and the IFP in the tumor were all measured on day 12. The antiangiogenic effects of TNP-470 were studied by scanning electron microscopic images of tumor vascular casts. TNP-470 was observed to inhibit tumor growth and increase the necrotic area significantly. In the TNP-470 treated group, the IFP in the superficial layer, defined as 2-3mm from the tumor capsule, and in the deep layer, defined as 8-10mm from the tumor capsule, were significantly higher than the corresponding values in the control.

Moreover, vascular casts showed a significant reduction in the budding of sprouts in the superficial layer, and a decrease in the maximum diameter of the tumor vessels in the deep layer. It is possible that the higher IFP in the TNP-470 treated tumors might have prevented tumor vessel dilation. The findings of this study demonstrated that TNP-470 inhibited the budding of tumor vessel sprouts, and increased the IFP. These processes seem to act synergistically to suppress tumor angiogenesis.

Introduction

It is well known that tumor growth requires both oxygen and nutrients, which are supplied through neovascularization. Angiogenic activity is therefore a prerequisite for the progression of tumors (1), and this observation has encouraged the development of antiangiogenic agents as a strategy in cancer therapy (2,3). Recently, TNP-470, a synthetic analog of fumagillin, an antibiotic isolated from Aspergillus fumigatus fresinius, was developed as an antiangiostatic agent (4). To date, there have been numerous studies regarding the inhibitory effects of TNP-470 on tumor growth and tumor angiogenesis (5,6,7,8,9,10). In fact, Morita et al. evaluated angiogenesis by quantifying the number of blood vessels using a dissecting microscope (11), and Yanase et al. stained tumor blood vessels with silver and scored the number of blood vessels morphometrically (12). Both studies showed that TNP-470 reduced the number of tumor vessels; however, no detailed studies on the effects of TNP-470 from the viewpoint of tumor vessel morphology have been performed.

The process of angiogenesis consists of many steps, including migration, the proliferation of endothelial cells, digestion of the extracellular matrix (ECM), and tube formation. These processes are partly influenced by the interstitial fluid pressure, which has been reported to be elevated in animal and human tumors (13,14,15). The elevated interstitial fluid pressure inside tumors is believed to have a strong influence on the formation of the tumor vasculature in rats inoculated subcutaneously (sc) with the Walker 256 carcinosarcoma cell line.

To investigate the mechanisms responsible for the effects of TNP-470, the tumor vessel architecture of animals injected with TNP-470 was compared to that of control animals by examining the ultrastructural alterations in the tumor vasculature and interstitial fluid pressure.

Materials and methods.

Materials

TNP-470, a synthetic analog of fumagillin which is an antibiotic isolated from Aspergillus fumigatus, was kindly donated by Takeda Chemical Industries Co., Ltd., Osaka, Japan. TNP-470 was suspended in a vehicle consisting of 1% ethanol and 5% gum arabic in normal saline.

Animals

Male Wistar rats weighing 200-250g were purchased from SLC Co.,Ltd., Shizuoka, Japan. Throughout the experimental period, the rats were fed commercial chow and maintained in an air-conditioned animal room. All rats were cared for in accordance with the criteria of the National Research Council.

Cell Line

The Walker 256 carcinosarcoma cell line was kindly donated by the Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University. This cell line, which was established from a rat mammary cancer, is subcutaneously implantable and rapidly exhibits a hypervascular network with a doubling time of 3.6 days.

Tumor Growth Assay and Measurement of the Interstitial Fluid Pressure in the Tumor

The rats were divided into a control group (n=40) and a TNP-470 treated group (n=40). Walker 256 carcinosarcoma cells, 1×10^7, were injected into the subcutaneous tissue in the inguinal region of the rats. In the control and TNP-470 treated groups, either the vehicle medium or TNP-470 at a dose of 30mg/kg body weight, respectively, was injected sc at a site remote from the tumor on day 1 after inoculation.

This protocol for the injection was determined by the fact that 30mg/kg body weight of TNP-470 is an established dose (9, 10, 11, 12), and because a stronger effect was expected by giving the injection earlier after the inoculation of tumor cells (12).

On day 12, the interstitial fluid pressure of the subcutaneous tumors was measured under diethyl ether anesthesia, after which the tumors were excised and their weight was recorded. To analyze the interstitial fluid pressure in relation to the tumor development, each group was subdivided into three groups according to tumor weight; namely, less than 2.5g, 2.5 g - 5.0g, and more than 5.0g.

The wick-in-needle technique was used to measure the interstitial fluid pressure (13,14,15). A number of 23-gauge needles (Terumo., Pharmacological., Ltd., Osaka , Japan) were prepared by placing three 5-0 monofilamentous nylon threads (Ethicon Endo-Surgery., Ltd., Osaka, Japan) in the barrels of the needles, and the bevels were curved and angled to avoid inserting a tissue core. At the time of interstitial fluid pressure measurement, the wick-in-needle was attached to a polyethylene tube connected to a pressure transducer. The polyethylene tube was flushed and filled with 0.9% normal saline containing 10u heparin in 1ml 0.9% normal saline, and the transducer signal was processed by a pressure transducer, a signal conditioner, and an amplifier (Bioresearch Systems, SEN-6102M, NIHON KOHDEN, Japan). The output was recorded on a strip chart recorder (Thermal Array Recorder, RTA-4100, NIHON KOHDEN, Japan). The system was periodically calibrated with a mercury manometer. At the time of measurement, the transducer diaphragm was placed at the same level as the tumor, and the fluid communication and system compliance were tested by clamping and unclamping the plastic tube between the wick-in-needle and the transducer. This was performed after the initial insertion of the needle. Following the induction of general anesthesia with diethyl ether, the rats were placed in a supine position.

The interstitial fluid pressure was measured at two points in the tumor; one in the superficial layer, defined as a depth of 2-3mm from the tumor capsule, and the other in the deep layer, defined as a depth of 8-10mm from the tumor capsule. After these measurements, each tumor was excised and its weight measured.

Measurement of the Necrotic Area in the Tumor.

Tissue specimens were obtained from rats sacrified on day 12 that had received either the control (n=20) or TNP-470 (n=20) on day 1 after the inoculation. Midline cross-sections through each tumor at the region of its greatest diameter were fixed with 10% formaldehyde, and stained with hematoxylin and eosin (HE). The ratio of the necrotic area(%) [necrotic area / total tumor area×100] was determined by an image analyzer (Cosmozone-1s; Nikon, Japan).

Tumor Vascular Casting

The rats were divided into a control group (n=15) and a TNP-470 treated group (n=15). In the control and TNP-470 treated groups, either vehicle or TNP-470 at a dose of 30mg/kg body weight, respectively, was injected at a site remote from the tumor on day 1. The rats were sacrified on day 12 and vascular casts were made as follows (16,17). Under ether anesthesia, a midline incision was made in the abdominal and pleural cavities of rats placed in the supine position. The thoracic aorta was dissected and cannulated using an 18G surflow catheter (Terumo), which was secured by ligation with 3-0 silk. The blood was then flushed from the vascular system by an infusion of a 0.9 % NaCl solution containing 10,000 u/l heparin and 5% saccharose (Wako., Pure., Chemical., Industries., Ltd., Osaka, Japan) given over 5 min.

A 2.5% glutaraldehyde solution (Wako, Ltd.) was then infused for 1 min, and an acrylic resin solution was prepared by mixing 20ml Mercox CL-2B (Okenshoji, Co.,Ltd., Tokyo, Japan), 5ml methylmetacrylate monomer (Wako, Ltd.), and 0.5g Catalyst-MA (Okenshoji, Co.,Ltd., Japan) in a 30ml-syringe, and infused via the aortic catheter at 120mmHg pressure until the Mercox CL-2B flowed out of the inferior vena cava. The resin was allowed to polymerize at room temperature for 15 min, then heated to 60° C for 15 min, and cooled to room temperature. The vascular casts were placed in a 20% NaOH solution at room temperature for 48 h to digest the tissue away. The casts were then rinsed thoroughly with distilled water at 60° C to remove the debris, and air dried at room temperature. The dried tumor casts were mounted on aluminium stubs, sputter-coated with gold (Eiko IB-3, ion coater) and the architecture of the blood vessels was examined with a scanning electron microscope (HITACHI S2250-N). The number of tumor vessels and sprouts, the maximum diameter of the tumor vessels at two points, and the superficial and deep layers from the tumor capsule were measured at five areas, randomly selected in each rat (n=15). Quantitative measurements were made using the automicrometer scale on the photographs.

Stastistical Analysis

The significance of differences between the experimental and control groups was calculated by the two-tailed Wilcoxon t test for unpaired values. A p -value< 0.05 was considered to be statistically significant.

Results

Tumor Growth Assay.

Of the tumors from the 40 control rats, 24 ranged in weight from 2.5g to 5.0g and the remaining 16 weighed more than 5.0g, whereas of those from the 40 TNP-470 treated rats, 15 weighed less than 2.5g and the remaining 25 ranged in weight from 2.5g to 5.0g. The mean tumor weights in the control and TNP-470 groups were 5.64±1.56g and 2.94 ±0.83g, respectively (p <0.05, Fig. 1). In the control group, all of the tumors weighed more than 2.5g, whereas in the TNP-470 group, they weighed less than 5.0g. Body weight was calculated before and after the experiment to evaluate the side effects. In the control group, the weight gain was 19.0±3.5%, while the weight loss in the TNP-470 treated group was 6.5±1.2%.

Interstitial Fluid Pressure in the Tumor

In the control group, the interstitial fluid pressures in the superficial layer of the tumors weighing 2.5g to 5.0g (n=24) and in those weighing greater than 5.0g (n=16) were 2.27±0.43mmHg and 3.78±0.61mmHg, respectively; the interstitial fluid pressures in the deep layer being 4.61±0.91mmHg and 6.84±1.26mmHg, respectively. In the TNP-470 treated group, the interstitial fluid pressures in the superficial layer of the tumors weighing less than 2.5g (n=15) and 2.5g - 5.0g (n=25) were 4.52±0.80mmHg and 5.14±1.02mmHg, respectively; the interstitial fluid pressures in the deep layer being 8.26±1.71mmHg and 8.99±1.66mmHg, respectively. The interstitial fluid pressures in the deep layer were significantly higher than those in the superficial layer in both groups, and those in the TNP-470 treated group were significantly higher than those in the control group in both layers of the tumors weighing 2.5-5.0g (p <0.05, Fig. 2).

Necrotic Areas in the Tumor

The ratio of the necrotic area in the tumors weighing 2.5g - 5.0g from the control group was 9.6±3.0%, versus 22.6±5.9% in those from the TNP-470 treated group (p <0.05, Fig. 3). Necrosis in the control group tumors occurred mainly in the central portion (Fig. 4A), while that in the TNP-470 treated group was scattered (Fig. 4B).

Tumor Vascular Casting

The numbers of tumor vessels in the superficial and deep layers of the control group tumors were 12±2 and 13±3 / one field at 500× magnification, respectively. In the TNP-470 treated group, the corresponding numbers were 9±2 and 10 ±3 / one field at 500× magnification, respectively (Table 1).

The maximum diameters of the tumor vessels in the superficial and deep layers of the control group tumors were 28±6 μm and 152±28 μm / one field at 500× magnification, respectively. In the TNP-470 treated group tumors, these values were 26 ±5 μm and 72±10 μm / one field at 500× magnification, respectively (Table 1).

The most remarkable difference between the control group and the TNP-470 treated group was observed in the superficial layer, where neovascularization is thought to be prevalent. In the control group tumors, the budding of numerous sprouts was observed up to 30±7 / one field at 500× magnification, whereas fewer sprouts of 4±2 / one field at 500× magnification were observed in the TNP-470 treated group (p <0.05, Fig. 5A,B,C,D). In the control group, the tumor vessels became dilated and tortuous, ultimately forming sinusoids in the deep layer of the tumors (Fig 6A). In contrast, in the TNP-470 treated group, the tumor vessels became partly dilated and partly stenotic (Fig 6B) as the interstitial fluid pressure increased in the deep layer. In both groups, few budding sprouts were observed in the deep layer of the tumors, without any significant difference (Table 1).

Discussion

Angiogenesis plays an important role in a variety of physiological processes, such as wound healing, corpus luteum formation and embryonic development, and also in pathological conditions such as diabetic retinopathy, arthritis and inflammation (18). The development of tumors also requires angiogenesis and therefore, the inhibition of angiogenesis in tumors could provide a novel strategy for the treatment of patients with cancer. The formation of new blood vessels is the result of a complicated process consisting of interactions between soluble angiogenic regulators, the insoluble extracellular matrix and extracellular matrix-degrading endothelial enzymes.

Fumagillin is a naturally secreted antibiotic produced by Aspergillus fumigatus fresenius, which inhibits the proliferation of human umbilical vein endothelial cells and tumor-induced angiogenesis in vivo. However, the administration of fumagillin has been limited in vivo, as it has been shown to cause severe weight loss and intestinal disturbances (4).

The recent development of TNP-470, one of the synthetic analogs of fumagillin, has attracted attention by demonstrating more potent antiangiostatic activity and less toxicity than fumagillin (19,20,21). The mechanisms responsible for the antiangiogenic activity of TNP-470 are thought to be related to the inhibition of signal transduction through c-fos and c-myc pathways in vascular endothelial cells (22), and many studies have reported the inhibitory actions of TNP-470 against tumor growth (6,7,8,9,10,11,12). Nevertheless, very few morphological and ultrastructural investigations of the tumor vessels have been performed in animals treated with TNP-470.

The vasculature of tumor tissue is characterized by a variety of structual alterations such as the continuous vascular remodeling of preexisting tumor blood vessels, the formation of new blood vessels, and the formation of new vascular networks with continuous vascular sprouting. The disorganized architecture of the vascular network thus reflects the chaotic growth of tumors (23). In this study, we observed morphological alterations in the tumor vessels as well as reduced tumor growth in rats injected with TNP-470. We confirmed that subcutaneous injections of TNP-470 at a dose of 30mg/kg body weight at a site remote from the tumor had a significant effect on tumor growth (8,9,10). Regarding side effects, a loss of weight was observed in the TNP-470 treated group, but no additional severe side effects were seen.

We also studied the influence of TNP-470 on tumor angiogenesis morphologically, using vascular casts, and evaluated these results in the context of the interstitial fluid pressure of these tumors, which is thought to have a potentially great influence on the tumor vasculature.

It has previously been reported that the interstitial fluid pressure in animal and human tumors is elevated, possibly due to the absence of functioning lymphatic circulation, clot formation in the tumor vessels, the high vascular permeability of tumor blood vessels, and blood vessel collapse (13,14,15). Boucher and Jain showed that the main driving force for this increased interstitial fluid pressure was the hydrostatic microvascular pressure of the postcapillary venules in the tumors, and that tumor interstitial hypertension was associated with the development of the tumor neovasculature (24,25).

The present study showed that the interstitial fluid pressure in the tumors weighing 2.5g - 5.0g from the TNP-470 treated rats was significantly higher than that of those from the control rats, in both the superficial and deep layers. The exact mechanisms

of the induction of increased interstitial fluid pressure by TNP-470 remain unknown. It is probable that absorption of the interstitial fluid may be impaired as a result of the microcirculatory disturbances caused by the suppression of tumor angiogenesis induced by TNP-470. In the present study, this led to a remarkable rise in the interstitial fluid pressure in the TNP-470 treated group. This increase in the interstitial fluid pressure acts synergistically on the inhibition of neovascularization, and also suppresses the budding of the sprouts.

While scanning electron microscopic analysis of the vascular casts clearly demonstrated the changes in vascular architecture induced by TNP-470, in this study some issues and discrepancies existed in these results. First, it was unclear why no statistical difference in the number of vessels was observed between the two groups despite a remarkable difference in the number of sprouts. We believe that the number of vessels and the number of sprouts did not always correlate because not all the sprouts grew to the vessels. Second, it was unclear why the number of sprouts in the deep layer of the tumors from the TNP-470 treated rats was same as that in those from the control rats, even though a great difference was observed in the superficial layer of the tumor from the two groups. It could be speculated that TNP-470 acted on the proliferating vascular endothelial cells, which existed preferentially in the superficial layer of the tumors. The third issue was why the maximum diameter was reduced only in the deep layer of the TNP-470 treated tumors. Our results showed a greater difference in interstitial fluid pressure between the two groups in the deep layer than in the superficial layer. Considering that the maximum diameter of the tumor vessels is regulated at least in part by the interstitial fluid pressure, this evidence may provide a partial explanation.

Finally, it should be noted that the rats in which the greatest reduction in tumor size was achieved by TNP-470 could not be used in this experiment, because the tumors less than 2.5g, on which TNP-470 was thought to exhibit the strongest effect, were not able to be compared to any of the control group tumors, all of which weighed more than 2.5g on day 12.

In conclusion, the findings of present study indicated that TNP-470 decreased the budding of sprouts in the vascular endothelia of tumor vessels and increased the intratumoral interstitial fluid pressure. Both these effects seemed to interact closely and possibly synergistically, thus playing a suppressive role in tumor angiogenesis.

Fig. 1 Tumor weights in the TNP-470 treated and control groups.

* $p < 0.05$ comparing the TNP-470 group with the control group.

Fig. 2 Interstitial fluid pressures in the superficial and deep layers of the control and TNP-470 treated tumors weighing 2.5g - 5.0g.

□ control ; ■ TNP-470 administered.

* $p < 0.05$ comparing the TNP-470 group with the control group.

Fig. 3 Ratio of the necrotic areas in tumors from the TNP-470 treated and control groups.

* $p < 0.05$ comparing the TNP-470 group with the control group.

Fig. 4 HE staining of (A) a control tumor, and (B) a TNP-470 treated tumor. The ratio of necrosis was as follows, (A) 9.8%, (B) 24.8%. The necrotic areas in the control tumor were located in the central portion, whereas those in the TNP-470 treated tumor were scattered throughout the tumor.

Fig. 5 Vascular casts in the superficial layer of a control tumor: (A)×200 and (B)×500; and a TNP-470 treated tumor: (C)×200 and (D)×500. The budding of sprouts was most prominent in the control tumor.

Fig. 6 Vascular casts in the deep layer of a control tumor: (A)×200, and a TNP-470 treated tumor: (B)×200. In the TNP-470 treated tumor, dilated vessels and a stenotic region (arrow) were both seen.

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	Superficial layer		Deep layer	
	Control	TNP-470	Control	TNP-470
Number of vessels (n=15)	12±2	9±2	13±3	10±3
Maximum vesssel diameter (n=15, μm)	28±6	26±5	152±28	72±10 *
Number of sprouts (n=15)	30±7	4±2 *	3±2	3±2

* $p < 0.05$ comparing the TNP-470 group with the control group

Table 1

Figure 1

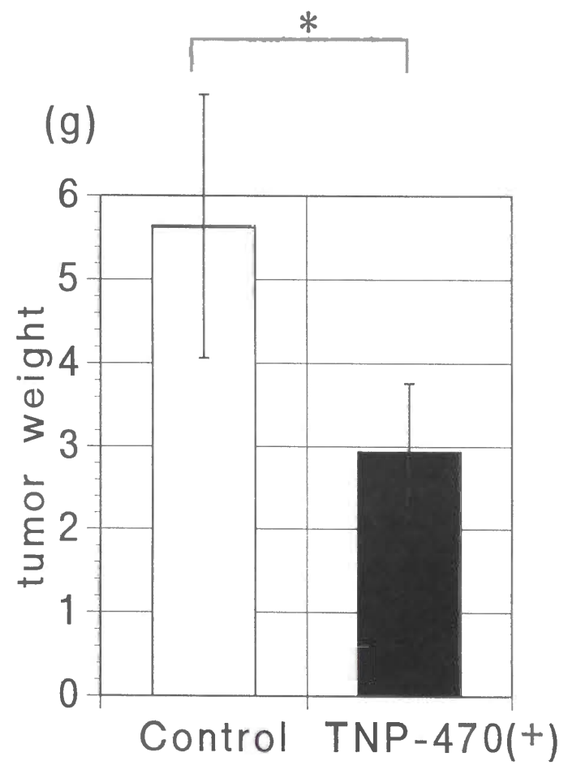
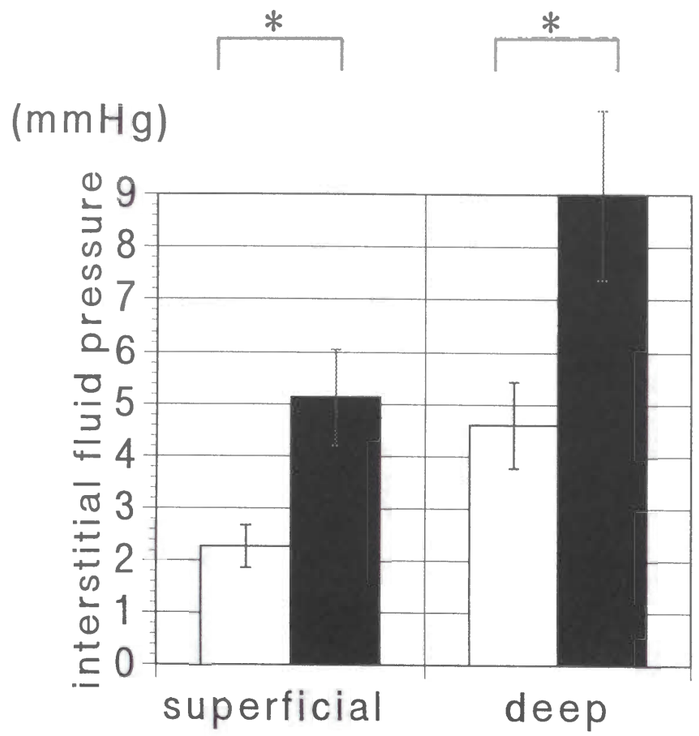


Figure 2



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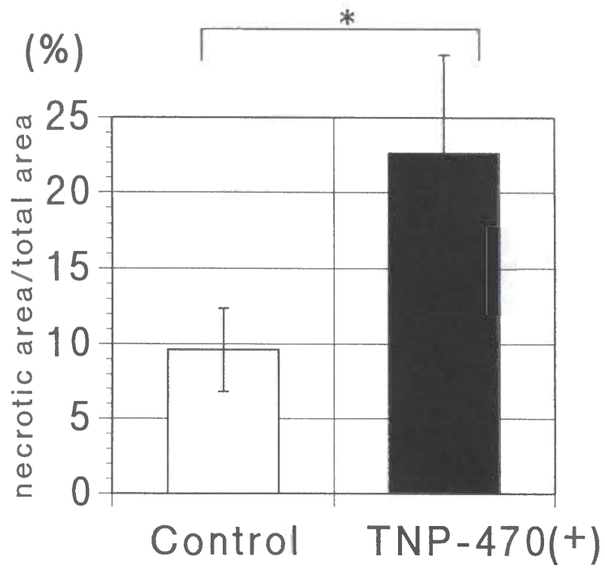

Figure 4A

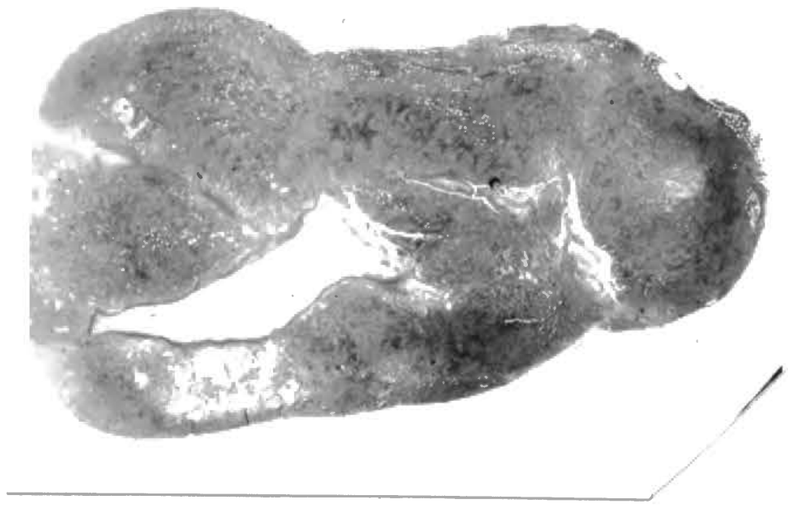

Figure 4B

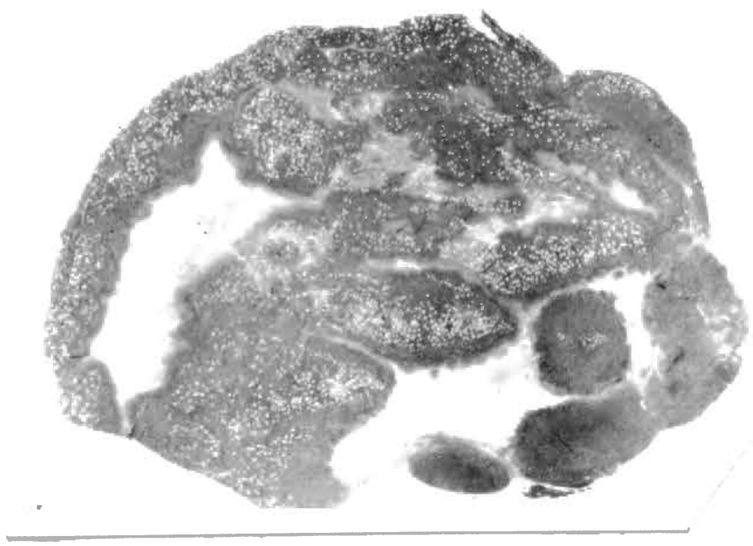

Figure 5A

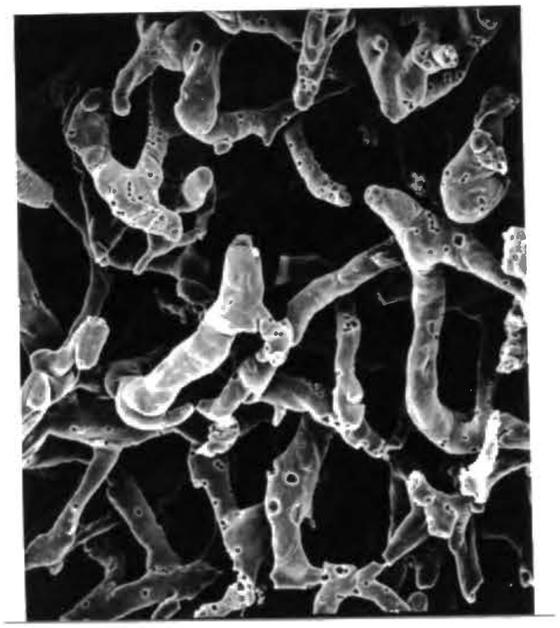

Figure 5B

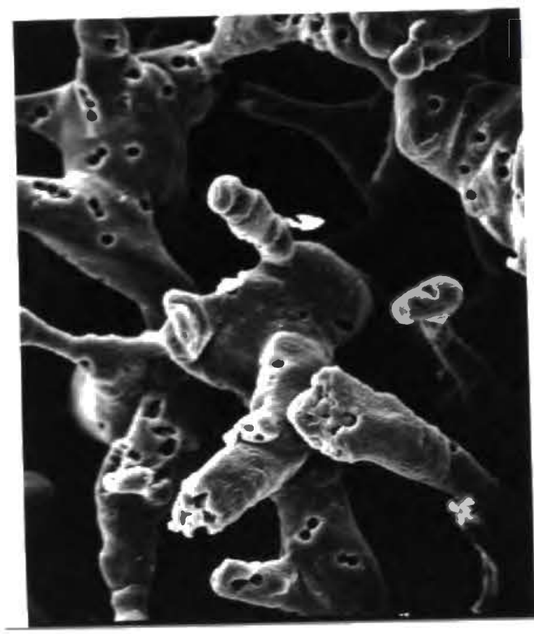

Figure 5C

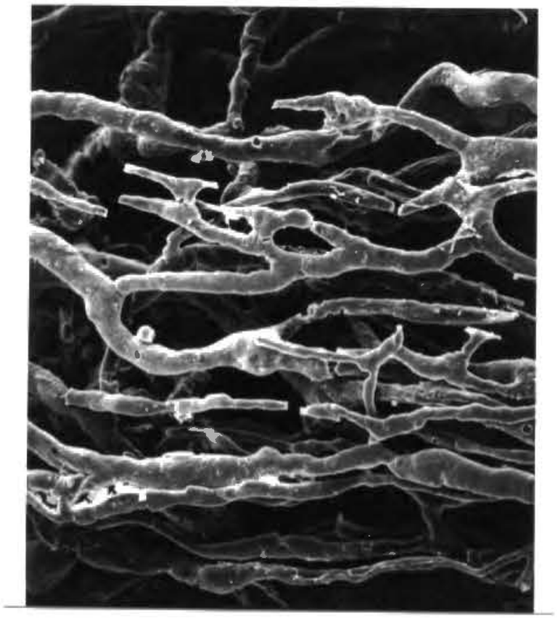

Figure 5D

Figure 6A

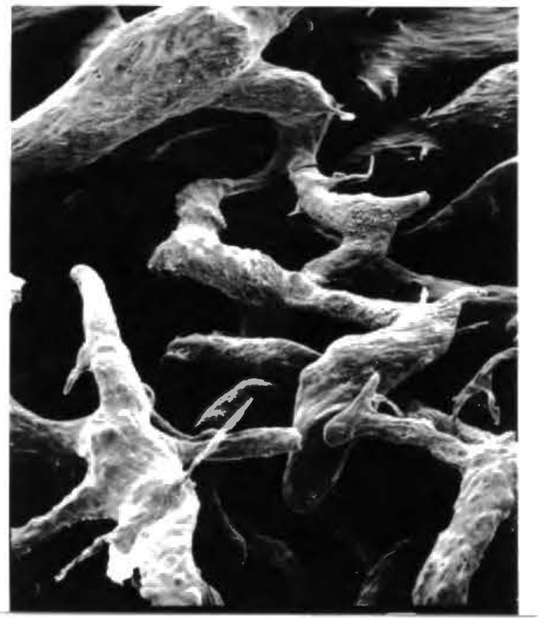

Figure 6B